Increasing cellulose production and transgenic plant growth in forest tree species

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Abstract: Cellulose is one of many important polymers in plants. Cellulose is made of repeat units of the monomer glucose. Cellulose is a major industrial biopolymer in the forest products, textile, and chemical industries. It also forms a large portion of the biomass useful in the generation of energy. Moreover, cellulose-based biomass is a renewable energy source that can be used for the generation of ethanol as a fuel. Cellulose is synthesized by a variety of living organisms such as plants and algae. It is the major component of plant cell walls with secondary cell walls having a much higher content of cellulose. The relationship between cellulose and lignin biosynthesis is complicated, but it is confirmed that inhibition of lignin biosynthesis in transgenic trees will increase cellulose biosynthesis and plant growth. Cellulose accumulation may be increased by down-regulating 4-coumarate:coenzyme A ligase (4CL, EC 6.2.1.12) as shown in transgenic aspen. There is no similar reports on down-regulating 4CL in transgenic conifers. Based on our established *Agrobacterium tumefaciens*-mediated transformation system in loblolly pine, we are able to produce antisense 4-CL transgenic loblolly pine which is predicted to have increasing cellulose accumulation. The overall objective of this project is to genetically engineer forest tree species such as loblolly pine with reduced amount of lignin and increased cellulose content. The research strategy includes: (1) isolate the 4-coumarate:coenzyme A ligase gene from loblolly pine seedlings by reverse transcription-polymerase chain reaction (RT-PCR) and Rapid Amplification of cDNA Ends-Polymerase Chain Reaction (RACE-PCR) techniques from the cDNA library; (2) construct binary expression vectors with antisense 4CL coding sequences and introduce antisense constructs of the 4-coumarate:coenzyme A ligase gene cloned from loblolly pine into the loblolly pine to down regulate the 4-coumarate:coenzyme A ligase gene expression; (3) study the effect of the antisense transgene expression on lignin content, cellulose accumulation, and loblolly pine biomass; and (4) select fast growth and high cellulose accumulation transgenic loblolly pine lines for future commercial application.

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Introduction

The biosynthesis of cellulose essentially proceeds by the polymerization of glucose residues using an activated substrate UDP-glucose (Amor *et al.* 1995; Arioli *et al.* 1998; Brown 1996). In plants, cellulose is synthesized on the plasma membrane by the enzyme cellulose synthase that is present in the membrane (Brown *et al.* 1997; Brummell *et al.* 1997; Carpita and Vergara 1998). In the alga *Pleurochrysis*, cellulose scales are formed in the Golgi apparatus and then deposited on the cell surface (Itoh 1990). Cellulose is an aggregate of glucan chains that are arranged in a specific manner to give rise to a crystalline state (Chanzy and Henrissat 1985; Delmer 1999). Although cellulose produced by different organisms has the same chemical composition (polymer of β-1,4- linked glucose residues), there are remarkable differences in the physical properties of the cellulose product, mainly in the length of the glucan chains (as represented by degree of polymerization) and the crystallinity and crystalline form of the cellulose product (Delmer 1987; Delmer and Amor 1995; Doblin *et al.* 2002). Depending upon the specific organism, this crystalline state is different, and it defines the physical properties of the product such as its strength, solubility in various solvents, and accessibility to various modifying reagents (Gardner and Blackwell 1974; Kawagoe and Delmer 1997; Lin and Brown 1989). Cellulose biosynthesis proceeds in at least two stages – polymerization and crystallization (Brown and Montezinos 1976; Carpita 1996; Delmer and Amor 1995). The first stage is catalyzed by the enzyme cellulose synthase, and the second stage is dependent on the organization of the cellulose synthases possibly with other proteins such that the glucan chains are assembled in a crystalline form (Pear *et al.* 1996; Thelen and Delmer 1986; VanderHart and Atalla 1986).

Significant progress has been made in recent years toward an understanding of lignin biosynthesis through characterization of biosynthetic pathway enzymes and genes from both herbaceous and tree crops (Christensen *et al.* 2000; Donaldson 2001; Jones *et al.* 2001; Neish 1968). Past efforts to reduce tree lignin content by downregulating genes encoding caffeate *O*-methyltransferase or cinnamyl alcohol dehydrogenase did not succeed, but instead modified lignin structure (Atanassova *et al.* 1995; Chen *et al.* 2000; Parvathi *et al.* 2001; Whetten *et al.* 1998, Zhong *et al.* 2000). This is in accordance with down regulating these genes in transgenic tobacco plants, suggesting that neither enzyme limits lignin accumulation. However, reduction of lignin in plants was demonstrated in transgenic tobacco by suppression of phenylalanine ammonia-lyase, an enzyme that catalyzes the entry step to the phenylpropanoid pathway upstream of lignin biosynthesis (Bolwell *et al.* 1986; Howles *et al.* 1996; Sewalt *et al.* 1997). Suppressing its activity restricts overall phenylpropanoid biosynthesis, resulting in a wide range of abnormal growth pheno-

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types as well as reduced thioglycolate-extractable lignin. Reduced lignin content was also achieved in transgenic tobacco and *Arabidopsis* by downregulating 4CL or cinnamoyl-coenzyme A reductase, but collapsed cell walls and stunted growth were reported in plants with the most severe lignin reductions (Blount *et al.* 2002; Ehlting *et al.* 1999; Franke *et al.* 2002; Kajita *et al.* 1997; Meyer *et al.* 1998). These herbaceous systems clearly illustrate the possibilities as well as the possible drawbacks of modifying secondary metabolism and lignin quantity in plants. Thus, the question from the perspective of wood biotechnology is whether lignin content can indeed be reduced without compromising the structural integrity and growth of trees.

The enzyme 4CL catalyzes the formation of CoA thioesters of cinnamic acids and is a key enzyme in lignin biosysthesis (Allina *et al.* 1998; Kajita *et al.* 1997). Transgenic aspen (*Populus tremuloides* Michx.) trees in which expression of a lignin biosynthetic pathway gene *Pt4CL1* encoding 4-coumarate:coenzyme A ligase (4CL) has been downregulated by antisense inhibition, demonstrated that trees with suppressed *Pt4CL1* expression exhibited up to a 45% reduction of lignin, but this was compensated for by a 15% increase in cellulose. As a result, the total lignin–cellulose mass remained essentially unchanged (Hu *et al.* 1999). These results indicate that lignin and cellulose deposition could be regulated in a compensatory fashion, which may contribute to metabolic flexibility and a growth advantage to sustain the long-term structural integrity of woody perennials (Abbott *et al.* 2002; Li *et al.* 2001). Secondary xylem (wood) of trees, from which pulp is derived, is composed of cellulose (β-1,4-glucan), lignin (phenolic polymer), and hemicelluloses (heterogeneous polysaccharides) in approximate proportions of 2:1:1. During tree growth, cellulose microfibrils give cell walls tensile strength, and lignin encasing the cellulose microfibrils imparts rigidity to cell walls (Anterola *et al.* 2002; Chen *et al.* 1999; Christensen *et al.* 2001; Dixon *et al.* 2001; Ranocha *et al.* 1999; Samuels *et al.* 2002). Regardless of its importance during growth, lignin becomes problematic to postharvest, cellulose-based wood processing, because it must be separated from cellulose at enormous energy, chemical, and environmental expense. As a result, there has been long-standing incentive to develop healthy trees that accumulate less lignin and higher cellulose to facilitate pulping (Sarkanen and Ludwig 1971; Osakabe *et al.* 1999). The research strategy includes: (1) use a cDNA library and reverse transcription-polymerase chain reaction (RT-PCR) and RACE-PCR strategies to clone the *4CL* gene from loblolly pine seedlings; (2) insert the full-length cDNA of *4CL* into a binary expression vector, pCAMBIA1301, and produce transgenic loblolly pine via *Agrobacterium tumefaciens*-mediated transformation; (3) determine and analyze the impact of expression of *4CL* on loblolly pine lignin content, cellulose accumulation, and biomass accumulation in different transgenic loblolly pine lines; and (4) select low lignin containing and high cellulose accumulating transgenic loblolly pine lines for future commercial application.

The 4-coumarate:coenzyme A ligase that catalyzes the CoA ligation of hydroxycinnamic acids and generates activated phenolic precursors for lignin and flavonoid biosynthesis was purified from differentiation xylem of loblolly pine (Grabber *et al.* 1997; Kajita *et al.* 1996; Voo *et al.* 1995). The pine enzyme had an apparent molecular mass of 64 kD and was similar in size and kinetic properties to 4CL isolated from Norway spruce. The cDNA clones for *4CL* were obtained from a xylem expression library. The cDNA sequence matched pine xylem 4CL protein sequences and showed 60 to 66% DNA sequence identity with a *4CL* sequence from herbaceous angiosperms (Voo *et al.* 1995; Whetten *et al.* 1998). The genetic analysis showed that 4-coumarate: coenzyme A ligase was from a single gene. By antisense downregulation of the lignin-specific *4CL* expression, Transgenic loblolly pine trees that accumulate structurally normal lignin at substantially reduced levels can be regenerated. The reduced lignin will be compensated for by a concomitant increase in cellulose. Our findings will confirm that the deposition of these two cell wall structural components in trees may be regulated in a compensatory fashion not reported in herbaceous plants. Furthermore, cell walls will not be collapsed; in fact, growth of transgenic plants will be substantially enhanced.

This research will utilize antisense constructs of the 4-coumarate: coenzyme A ligase gene to regulate lignin biosynthesis in loblolly pine. We have accomplished the following specific objectives: (1) we have developed a high-efficiency protocol for *Agrobacterium*-mediated gene transformation and regeneration for loblolly pine using different families provided by the Tree Improvement Program at North Carolina State University (Tang et al. 2001); (2) the 4-coumarate:coenzyme A ligase gene has been cloned (Voo et al. 1995), sequenced (GenBank accession # U12012), and inserted into a binary expression vector, pBIN-mGFP5-ER or/and pCAMBIA 1301; (3) using our protocol for transformation and regeneration, we have produced transgenic loblolly pine plants derived from seven different families. Transformation of loblolly pine with antisense constructs of the 4-coumarate:coenzyme A ligase gene using *Agrobacterium tumifaciens* will be achieved using our developed protocol. Regeneration of transformants and selection of transgenic loblolly pine plants of various phenotypes will be investigated for regulation of indigenous 4-coumarate: coenzyme A ligase gene expression which includes Southern and Northern analysis, 4-coumarate:coenzyme A ligase enzyme assays, and determination of lignin and cellulose contents from developed woody tissue.

Relationship between lignin and cellulose biosynthesis

It is interesting to note that benzodioxane J structures, which are found in plants with suppressed *COMT* expression, are also present in the lignin of *F5H*-overexpressing *Arabidopsis* plants, probably because of the increased flux to 5-hydroxyconiferaldehyde and 5-hydroxyconiferyl alcohol M15H without an associated increase in *COMT* activity (Chapple 1998). These data show that F5H plays a major role in 5-hydroxylation and that it is possible to modulate the S/G ratio in plants from one extreme to the other. Although CAD catalyzes the last step in monolignol biosynthesis, lignin content is only slightly affected in most CAD-deficient plants (Baucher *et al.* 1999; Hibino *et al.* 1995; Mackay *et al.* 1997; Zubieta *et al.* 2002). This can, at least in part, be explained by the incorporation of other phenolics that compensate for the reduced availability of monolignols for polymerization. Indeed, downregulation of CAD in tobacco and poplar results in conifer aldehyde and sinapaldehyde incorporation into the lignin polymer (Keller *et al.* 1989). In pine, a CAD mutation has been associated with an increased amount of coniferaldehyde X2G and unanticipated dihydroconiferyl alcohol X5G units (Ralph *et al.* 1997; Sederoff *et al.* 1999). The CAD deficient *bm1* mutant of maize has a 20% reduced lignin contents (Halpin *et al.* 1998) and stains more

strongly for aldehydes; however, no aldehyde resonances were seen in nuclear magnetic resonance spectra (Mackay *et al.* 1997).

The expression of two or three genes of the monolignol biosynthetic pathway has been altered either by crossing single transformants downregulated for particular genes (Chabannes *et al.* 2001a), by double transformation (Guo *et al.* 2001; Zhong *et al.* 1998), or by introduction of a chimeric construct consisting of fragments of three genes (Abbott *et al.* 2002). A striking characteristic of transgenic plants down-regulated for 4CL, CCoAOMT, CCR, CAD, and COMT is the reddish or brownish discoloration of the xylem tissues, initially observed in the maize "*brown-midrib*" (*bm*) mutants. The reddish coloration of CAD- and COMT deficient plants has been attributed to the incorporation of cinnamaldehydes in the polymer; synthetic DHPs of coniferyl alcohol and conifer aldehyde also form a red polymer (Hibino *et al.* 1995). However, in plants down-regulated for 4CL, CCoAOMT, and CCR, the incorporation of other phenolics into the lignin polymer is the likely cause of the xylem discoloration (Boerjan *et al.* 2003).

Results obtained by analyzing transgenic plants modified in monolignol biosynthesis have demonstrated that plants can tolerate large variations in lignin content and composition, that monomers other than *p*-coumaryl, coniferyl, and sinapyl alcohol are incorporated into the lignin polymer, and that the copolymerization of these uncommon monomers may result in novel lignin structures (Boerjan *et al.* 2003). These data show that the lignin polymer is extremely flexible in its composition. Plants down-regulated in C3H, CCoAOMT, and CCR all had reductions in lignin content associated with a collapse of the vessels and altered growth, phenotypes that may significantly vary according to developmental, and they have been shown to have environmental conditions (Boerjan *et al.* 2003; Whetten *et al.* 1998; Humphreys *et al.* 1999). Such collapsed vessels have been studied in detail in CCR-downregulated tobacco and *Arabidopsis irx4* mutants and have an expanded S2 secondary wall with individualized cellulose microfibrils (Chabannes *et al.* 2001), indicating an important role for lignin as a cohesive compound between cellulose microfibrils. However, in some cases, reduced lignin content is not associated with growth abnormalities (Lee *et al.* 1997; Humphreys *et al.* 1999; Marita *et al.* 1999). In the case of 4CL downregulation in aspen with increased growth, lignin content per se were not essential for structural integrity of the cell wall and that the reduced lignin content can be compensated by other cell wall constituents (Hu *et al.* 1999). The increase in cellulose coupled to a decrease in lignin observed in 4CL-downregulated poplars, is predicted to be similar in transgenic pines.

Agrobacterium-mediated genetic transformation system in Loblolly Pine

An engineered green fluorescent protein (*m-gfp5-ER*) gene under the control of the 35S Cauliflower Mosaic Virus promoter was used to develop a facile and rapid loblolly pine (*Pinus taeda* L.) transformation system via *Agrobacterium tumefaciens*-mediated transformation of mature zygotic embryos. Green fluorescent protein has been introduced into three different loblolly pine families that are considered recalcitrant to transformation. The *m-gfp5-ER* gene produced bright-green fluorescence easily detectable and screenable in loblolly pine tissue 3–30 days after explants were co-cultivated with *Agrobacterium*. A high-level of GFP expression was detected in transgenic cells, tissues, and plants, and was localized in specific cells derived from cotyledons, hypocotyls, and radicles of mature zygotic embryos. Furthermore, in vitro and in vivo monitoring of GFP expression permitted a rapid and easy discrimination of transgenic shoots, and drastically reduced the quantity of tissue to be handled and the time required for the recovery of transformed plants. Integration of the *m-gfp5-ER* was confirmed by polymerase chain reaction (PCR), by Southern and northern blot analysis, and by junction DNA sequence analysis. Molecular analysis of *Agrobacterium* T-DNA loci in transgenic loblolly pine demonstrated that most of the transgenic plants were derived from single transformation events. GFP-expressing shoots were also observed in loblolly pine explants co-cultivated with *Agrobacterium* but cultured in a medium without the selective agent kanamycin. This provides the opportunity to regenerate transgenic plants without using selectable-marker antibiotic-resistance genes, which will enhance the commercialization of transgenic plants (Tang *et al.* 2001).

Loblolly pine (*Pinus taeda* L.) is an economically important coniferous species that is widely planted in southern U.S. Both in vitro regeneration system of loblolly pine via somatic embryogenesis or organogenesis and regeneration system of transgenic plants have been established in East Carolina University and North Carolina State University. The Tree Improvement Program of North Carolina State University has nearly 50 years of experience in loblolly pine genetic improvement and has produced special elite families with superior growth for this research. If transgenic loblolly pine expressing the *4CL* gene can reduce lignin content by 45% and increase cellulose accumulation by 15% like transgenic aspen, we will be able to shorten the harvest period about 5 years. This will save millions of dollars for industrial companies each year. Our future study will be conducted to examine the effects of expressing a loblolly pine *4CL* gene linked to a constitutive CaMV 35S promoter, a phenylalanine ammonia lyase (PAL) promoter, and a caffeoyl CoA 3-O-methyltransferase (CCOMT) promoter in loblolly pine trees, and to produce superior transgenic loblolly pine with tissue-specific expression of *4CL*. Our long-term purpose is to produce fast growing loblolly pine strains based on the improved cellulose accumulation and increased biomass, and to make these transgenic lines available to the forest industry.

Strategy to genetically engineer forest tree species loblolly pine

The overall objective of this project is to genetically engineer forest tree species loblolly pine with a reduced amount of lignin and an increased content of cellulose by down regulating 4-coumarate:coenzyme A ligase gene expression in transgenic plants. The research strategy includes: (1) using reverse transcription-polymerase chain reaction (RT-PCR) and Rapid Amplification of cDNA Ends-Polymerase Chain Reaction (RACE-PCR) techniques to isolate the 4-coumarate:coenzyme A ligase (4CL: EC6.2.1.12) cDNA coding region from loblolly pine seedlings. This technique is used to obtain the 3'end of a cDNA, and it requires sequence information internal to the mRNA under study. The sequence information obtained from this technique can be utilized to obtain full length cDNA clones using the 5'RACE technique. The loblolly pine 4CL cDNA sequence (GenBank accession No. U12012) is as follows: 5'-ctcattcaat tcttcccact gcaggctaca tttgtcagac acgttttccg ccatttttcg

cctgtttctg cggagaattt gatcaggttc ggattgggat tgaatcaatt gaaaggtttt tattttcagt atttcgatcg ccatggccaa cggaatcaag aaggtcgagc atctgtacag atcgaagctt cccgatatcg agatctccga ccatctgcct cttcattcgt attgctttga gagagtagcg gaattcgcag acagaccctg tctgatcgat ggggcgacag acagaactta ttgcttttca gaggtggaac taatttctcg caaggtcgct gccggtctgg cgaagctcgg gttgcagcag gggcaggttg tcatgcttct ccttccgaat tgcatcgaat ttgcgtttgt gttcatgggg gcctctgtcc ggggcgccat tgtgaccacg gccaatcctt tctacaagcc gggcgagatc gccaaacagg ccaaggccgc aggcgcgcgc atcatagtta ccctggcagc ttatgttgag aaactggccg atctgcagag ccacgatgtg ctcgtcatca caatcgatga tgctcccaag gaaggttgcc aacatatttc cgttctgacc gaagccgacg aaacccaatg cccggccgtg aaaatccacc cggacgatgt cgtggcgttg ccctattctt ccggaaccac ggggctcccc aagggcgtga tgttaacgca caaaggcctg gtgtccagcg ttgcccagca ggtcgatggt gaaaatccca atctgtattt ccattccgat gacgtgatac tctgtgtctt gcctcttttc cacatctatt ctctcaattc ggttctcctc tgcgcgctca gagccggggc tgcgaccctg attatgcaga aattcaacct cacgacctgt ctggagctga ttcagaaata caaggttacc gttgccccaa ttgtgcctcc aattgtcctg gacatcacaa agagccccat cgtttcccag tacgatgtct cgtccgtccg gataatcatg tccggcgctg cgcctctcgg gaaggaactc gaagatgccc tcagagagcg ttttcccaag gccattttcg ggcagggcta cggcatgaca gaagcaggcc cggtgctggc aatgaaccta gccttcgcaa agaatccttt ccccgtcaaa tctggctcct gcggaacagt cgtccggaac gctcaaataa agatcctcga tacagaaact ggcgaatctc tcccgcacaa tcaagccggc gaaatctgca tccgcggacc cgaaataatg aaaggatata ttaacgaccc ggaatccacg gccgctacaa tcgatgaaga aggctggctc cacacaggcg acgtcgaata cattgacgat gacgaagaaa tcttcatagt cgacagagta aaggagatta tcaaatataa gggcttccag gtggctcctg ctgagctgga agctttactt gtggctcatc cgtcaatcgc tgacgcagca gtcgttcctc aaaagcacga ggaggcgggc gaggttccgg tggccttcgt ggtgaagtcg tcggaaatca gcgagcagga aatcaaggag ttcgtggcaa agcaggtgat tttctacaag aaaatacaca gagtttactt tgtggatgcg attcctaagt cgccgtccgg caagattctg agaaaggatt tgagaagcag actggcagca aaatgaaaat gaatttccat atgattctaa gattcctttg ccgataatta taggattcct ttctgttcac ttctatttat ataataaagt ggtgcagagt aagcgcccta taaggagaga gagagcttat caattgtatc atatggattg tcaacgccct acactcttgc gatcgcttca atatgcatat tactataaac gatatatgtt ttttttttt-3' (Start codon: ATG, Stop codon: TGA, Amino acid: 538); (2) Expression vector construction: After the 4Cl coding sequence is cloned and purified from the loblolly pine cDNA library, the sequence will be inserted in antisense in expression vector pBIN-mGFP5-ER by replacing the mGFP5-ER fragment or/and pCAMBIA 1301. The plasmid vector will be introduced into *Agrobacterium tumefaciens* LBA4404 by electroporation. Then introduce antisense constructs of 4-coumarate:coenzyme A ligase gene cloned from loblolly pine into loblolly pine to down regulate the 4-coumarate:coenzyme A ligase gene expression; (3) *Agrobacterium tumefaciens*-mediated transformation: An *Agrobacterium tumefaciens*-mediated transformation system (Tang *et al.* 2001) of mature zygotic embryos in loblolly pine has been established by using green fluorescent protein as a reporter gene. The successful transformation has been completed in three different loblolly pine families that are considered recalcitrant to transformation. Integration of the *m-gfp5-ER* was confirmed by polymerase chain reaction (PCR), by Southern and northern blot analysis, and by junction DNA sequence analysis. Molecular analysis of *Agrobacterium* T-DNA loci in transgenic loblolly pine demonstrated that most of transgenic plants were derived from single transformation events. The same transformation protocol will be used in this project to ensure the success of 4CL gene transfer into loblolly pine; (4) study the effect of antisense transgene expression on lignin content, cellulose accumulation, and loblolly pine biomass; and (5) select fast growing and high cellulose accumulating transgenic loblolly pine strains for future commercial application.

Anticipated Economic and Environmental Benefits

Loblolly pine (*Pinus taeda* L.) is an economically important coniferous species that is widely planted in southern U.S. As cellulose is a major component of its biomass, our overall goal is to utilize our knowledge of lignin and cellulose biosynthesis to improve cellulose production in loblolly pine. Transgenic loblolly pine with modified lignin content and cellulose accumulation is useful in a number of industrial applications. There are several important benefits to be gained by expressing the antisense constructs of the 4-coumarate:coenzyme A ligase gene cloned from loblolly pine into the loblolly pine. By down-regulating the 4-coumarate:coenzyme A ligase gene, it is hypothesized that transgenic trees will enhance cellulose production, decrease lignin content, and overcome problems of cellulose-based wood processing. Because of high lignin content in non-transgenics, it can be separated from cellulose at enormous energy, chemical, and environmental expense. This kind of research will lead to the production of commercially important transgenic loblolly pine.

Introducing antisense constructs of the loblolly pine 4-coumarate:coenzyme A ligase gene clone into loblolly pine to regulate the 4-coumarate:coenzyme A ligase gene expression will lead to the production of transgenic plants with low lignin content, high cellulose accumulation, and increased biomass. The fast growing and high cellulose accumulation transgenic loblolly pine lines will be available for future commercial application. There are several important insights to be gained by expressing antisense constructs of loblolly pine 4-coumarate:coenzyme A ligase: (1) this research will be the first attempt to genetically engineer lignin and cellulose biosynthesis by using loblolly pine as a model species in conifers; (2) the production of transgenic plants with low lignin content, high cellulose accumulation, and increased biomass will benefit forest industries by overcoming the problems of cellulose-based wood processing whereby lignin can be separated from cellulose at only enormous energy, chemical, and environmental expense.

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